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Identification of Antigenic Differences Between the Phosphorylated and Nonphosphorylated Forms of the E7 Protein of Human Papillomavirus Type 16

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To analyze the antigenic properties of the human papillomavirus type 16 E7 oncoprotein, two monoclonal antibodies, VD6 and IB10, that have different reactivities to the E7 protein were generated. While the VD6 antibody reacted strongly with E7 protein in CaSki cell extracts, the other antibody, IB10, showed much weaker reactivity with E7. This reactivity increased in a dose-dependent manner in the presence of the casein kinase II-specific inhibitor DRB (5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole). Antigenic site estimation and an in vitro phosphorylation assay, using bacterially expressed E7 protein, demonstrated that the weak reactivity of IB10 was related to the phosphorylation status of the E7 protein. Phosphorylation of E7 reduced considerably the reactivity of IB10 but did not affect the reactivity of VD6, which reacts with the N-terminal portion of E7. In immunoprecipitation (IP) assays, IB10 precipitated weakly the E7 protein from CaSki cell extracts. Together, these data suggest that unphosphorylated E7 protein shows distinct antigenic character compared to its phosphorylated form under denaturing conditions; however, under native conditions, the phosphorylated and nonphosphorylated E7 proteins have some antigenic cross-reactivity. ***J. Med. Virol. 54:129–134, 1998.***

KEY WORDS: human papillomavirus; E7; monoclonal antibody; antigenesity

INTRODUCTION

The E7 gene of the cancer-associated human papillomavirus (HPVs) encodes an important oncogene. HPV-16 and HPV-18 selectively retain and express the E7 gene in cervical carcinomas and cell lines derived from them [Macnab et al., 1986, Baker et al., 1987]. Numerous reports describe the oncogenic potential of the E7 gene [Edmonds and Vousden, 1989; Hawley-Nelson et al., 1989], and several have described the presence of antibodies to the E7 protein in sera from patients with cervical cancer [Jochmus-Kudielka et al., 1989; Kanda et al., 1992]. Anti-E7 antibodies are found more frequently in cervical cancer patients than in matched normal controls suggesting that the generation of anti-E7 antibody may be related to the development of cervical cancer [Krchnak et al., 1990; Muller et al., 1992]. The major immunogenic region of E7 is thought to extend from the N-terminus, through the Rb binding site and casein kinase II (CKII) phosphorylation sites, to the start of the proposed zinc finger sites [Krchnak et al., 1990; Dillner, 1990; Stacey et al., 1994]. However, the precise immunogenic regions within the CRII region of E7 have yet to be clearly defined.

The E7 protein is known to be modified after translation by CKII at serine residues 31 and 32 in the CRII region [Barbosa et al., 1990; Firzlaff et al., 1991]. This phosphorylation could affect the antigenicity of E7. We examined this possibility by using a monoclonal antibody that reacts with the possible phosphorylation sites of the E7 protein.

MATERIALS AND METHODS

Construction of E7 Expression Plasmids and Purification of Expressed Proteins

The HPV16 E7 gene was obtained from the pHPV16 plasmid (ATCC No. 45113) by using PCR amplification. The oligonucleotide primers (E7-USP and E7-DSP) used to PCR amplify the E7 open reading frame (ORF) were designed to amplify E7 from the start to the stop codon and to allow in-frame directional cloning. Restriction enzyme recognition sequences were attached to the 5′ end of each primer sequence. The sequence of primer E7-USP was 5′-CTC GGATCC ATGCA TGGAG

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TABLE I. Plasmids Used

Name[1]	Expression region of E7	size (kDa)[2]	Use
pIH-E7	start to stop maltose binding protein (MBP) fusion	about 65	immunizing antigen
pEX-E7	start to stop T7-gene 10 fusion	about 45	immunizing antigen
pIH-821	MBP and α-fragment fusion deletion mutants[3]	about 50	negative control
p5d-1	from 2nd His to stop	about 65	antigenic site estimation
p5d-7	from 7th Thr to stop	about 63	antigenic site estimation
p5d-15	from 15th Leu to stop	about 60	antigenic site estimation
p5d-18	from 18th Glu to stop	about 55	antigenic site estimation
p3d-88	from start (Met) to 88th Gly	about 65	antigenic site estimation
p3d-79	from start (Met) to 79th Leu	about 64	antigenic site estimation
p3d-66	from start (Met) to 66th Arg	about 65	antigenic site estimation
p3d-44	from start (Met) to 44th Gln	about 58	antigenic site estimation
p3d-36	from start (Met) to 36th Asp	about 50	antigenic site estimation
p3d-33	from start (Met) to 33rd Glu	about 50	antigenic site estimation

[1]p5d series plasmids were 5′ deletion mutants and p3d series were 3′ deletion mutants.
[2]The sizes of expressed protein size from E7 proteins were the sum of MBP (about 45 kDa) and E7. The sizes of deletion mutants were not consistent with the deleted E7 gene size because the bidirectional deletion resulted that some part of MBP was deleted in case of 5′ deletion and some coding region was added to 3′ side of 3′ deleted E7 gene.
[3]The deletion plasmids were produced from pIH-E7 plasmid.

ATACA-3′ (*Bam*HI) and that of E7-DSP was 5′-CTC GGATCC AAGCTT GATCA GCCA-3′ (*Bam*HI and *Hind*III). The amplified HPV 16 E7 ORF was introduced into pIH821 (New England Biolabs, USA) and pGEMEX-1 (Promega, USA) to generate fusion proteins with the maltose binding protein (MBP) and the T7 phage gene 10 product, respectively (Table I). The E7 fusion proteins expressed were purified by amylose affinity chromatography (MBP fusion form) (New England Biolabs, USA) or by electrophoretic elution (gene 10 fusion form) (Prep cell, Bio-Rad, USA) according to the manufacturer's instructions.

Generation of Monoclonal Antibodies

BALB/c mice were immunized four times at 2-week intervals with 50 μg MBP-E7 (first two immunizations) and gene 10-E7 (last two immunizations). The fusion proteins were given intraperitoneally, and the immunizations were boosted intravenously 4 days prior to the removal of the spleen. The spleen was removed and dissociated into spleen cells, which were then fused with the P3-x63-Ag8.653 myeloma cell line by the standard method [Lindell and Cryer, 1991]. Of the hybridomas positive for MBP-E7 but negative for MBP alone in the ELISA screen, stable cell lines were established from two clones (VD6 and IB10). The resulting monoclonal antibodies (Mab) were isotyped (Mouse monoclonal sub-isotyping kit, HyClone, USA) and purified by protein A agarose affinity chromatography (MAPSII, Bio-Rad, USA).

Deletions

5′ to 3′ or 3′ to 5′ deletions were made in the E7 gene of the expression plasmid pIH-E7 (Table I). The deletions were made using exonuclease III as described by Henikoff [1987]. Briefly, the pIH-E7 plasmid was linearized by digestion with *Bam*HI to create the 5′ to 3′ deletion with *Hind*III for the 3′ to 5′ deletion. The absence of unique restriction enzyme sites on either side of the E7 gene led to the bidirectional exo III deletion. In this case, the 5′ and the 3′ sides of the DNA insert in pIH821 were also deleted. These linearized plasmids were then digested by exonuclease III at 20°C for the appropriate time intervals. S1 nuclease and Klenow fragment were then used to blunt-end the plasmids. These plasmids were then self-ligated and used to transform *E. coli*. Appropriate protein expression was confirmed by SDS-PAGE and Coomassie blue staining.

Western Blot Analysis and Immunoprecipitation

The CaSki cell line (ATCC No. CRL 1550) was used as a source of eukaryotically expressed E7 protein. Bacterial lysates or CaSki cell lysates were separated by 10% or 15% SDS-PAGE, transferred to nitrocellulose membranes, and stained with antibodies as described previously [Bollag and Edelstein, 1991]. The antibody-reactive bands were visualized by using chemiluminescence substrates (ECL, Amersham, UK) or the BCIP/NBT system (Immunoselect, Stratagene, USA) with the appropriate secondary antibodies. In some cases, the electrophoresed gel was soaked in 0.5% Coomassie brilliant blue solution to visualize the protein bands. The inhibition of intrinsic casein kinase II activity was attempted by adding DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) [Zandomeni and Weinmann, 1984] to the CaSki cell cultures 2 hours before the cells were lysed. The DRB-treated CaSki cell lysates were subjected to Western blot analysis to evaluate the reactivities of VD6 and IB10. Immunoprecipitation was carried out as described previously [Smootkin and Wettstein, 1987] with some modifications. The CaSki cultures, in 100 mm dishes, were harvested in RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1%

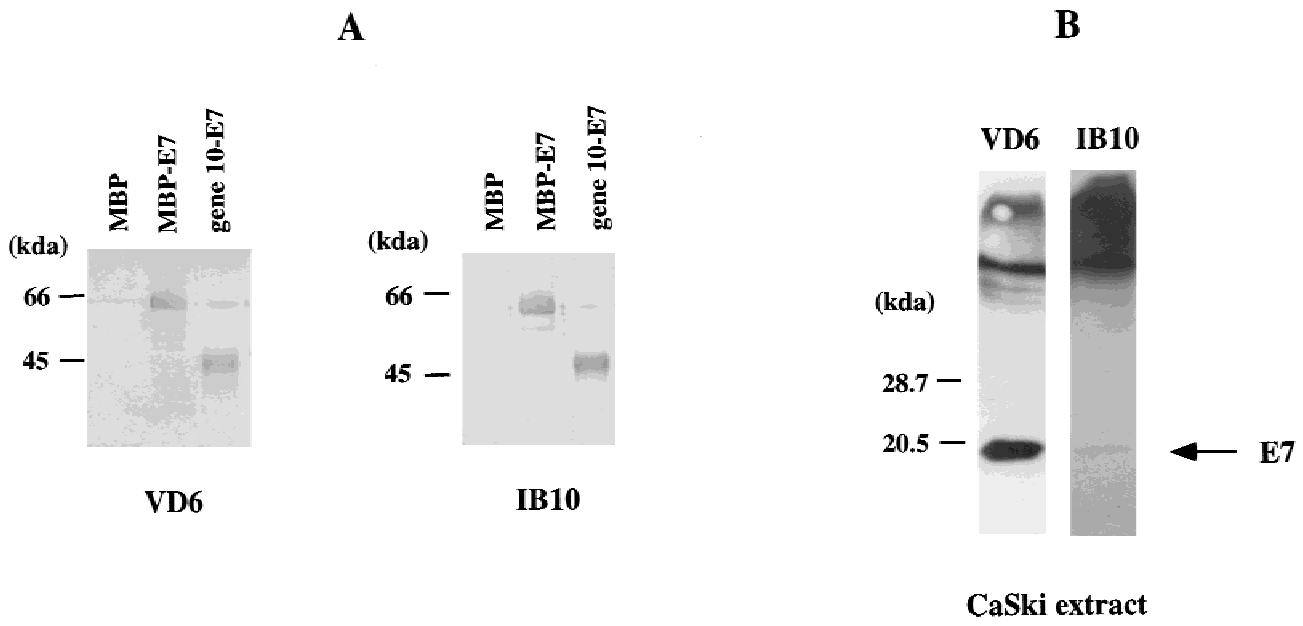

Fig. 1. Western blot analysis of E7 expression in bacterial extracts **(A)** and CaSki extracts **(B)** using VD6 and IB10. The sizes of the molecular weight standards are indicated to the left of each figure.

Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) and centrifuged to obtain supernatant. This supernatant was precleared with normal mouse sera and then mixed with VD6, IB10, or normal mouse antisera (negative control) (5 μg each). Immune complexes were precipitated with protein A-agarose (Bio-Rad, USA), washed five times with RIPA buffer, and then subjected to SDS-PAGE and Western blot analysis.

In Vitro Phosphorylation

The in vitro phosphorylation assay was carried out as described previously [Barbosa et al. 1990]. Purified MBP-E7 fusion protein (500 ng) was mixed with casein kinase II (Boeringer Mannheim, Germany) and [γ-^{32}P] ATP (5 μCi, 3000 Ci/mmol) in 50 μL reaction buffer (20 mM Hepes, 130 mM KCl, 10 mM $MgCl_2$, 4,8 mM dithiothreitol) and incubated at 30°C for up to 30 min. The samples were then subjected to SDS-PAGE and autoradiography. These reaction mixtures were also examined by Western blot analysis with the IB10 and VD6 antibodies. The results were quantitated by using an image densitometer (Molecular Dynamics, USA).

RESULTS

Production of Monoclonal Antibodies

To generate monoclonal antibodies, the bacterial expression vectors pIH821 and pGEMEX-1 were used to express E7 fusion proteins (a maltose binding protein, MBP and a T7-gene 10 fusion protein, respectively). These E7 fusion proteins were purified and used to immunize Balb/c mice. Two hybridoma clones, VD6 and IB10, were established by standard hybridoma techniques. These hybridoma cell lines secreted monoclonal antibodies (both of which were subtype IgG2a) that reacted strongly to both fusion proteins (MBP-E7 and gene 10-E7) but did not react to MBP alone, as determined by ELISA and by Western blot analysis (Fig. 1A). Western blot analysis using whole cell extracts of the CaSki cell line revealed that the reactivity of VD6 to E7 protein was relatively strong compared to that of IB10 (Fig. 1B). The strong reactivity of IB10 to bacterially expressed E7 protein suggested to us that factors other than E7 conformation could be responsible for the weak reactivity of IB10 to E7 protein expressed in CaSki cells. Treating the CaSki cultures for 2 hours with DRB increased the reactivity of IB10 with E7 in dose-dependent manner, but did not affect the reactivity of VD6 (Fig. 2). DRB inhibits in vivo and in vitro phosphorylation by CKII [Zandomeni and Weinmann, 1984]. Because E7 is known to be phosphorylated by CKII at serine residues 31 and 32 [Barbosa et al., 1990; Firzlaff et al., 1991], it was reasoned that the weak reactivity of IB10 could be related to the phosphorylation of E7.

Estimation of Antigenic Sites of Mabs

To estimate the antigenic sites of our monoclonal antibodies (VD6 and IB10), ten E7 deletion mutants were constructed using exonuclease III (Table I). The deletions were confirmed by DNA sequencing. Dot blot immunoassays with these deletion mutants showed that the reactivities of VD6 were maintained in all of the 3′ deletion proteins (p3d-88, p3d-79, p3d-66, p3d-44, p3d-36, and p3d-33), but that VD6 reactivities with all but one of the 5′ deletion mutants were lost. The sole 5′ mutant that remained antigenic (p5d-1) had lost a

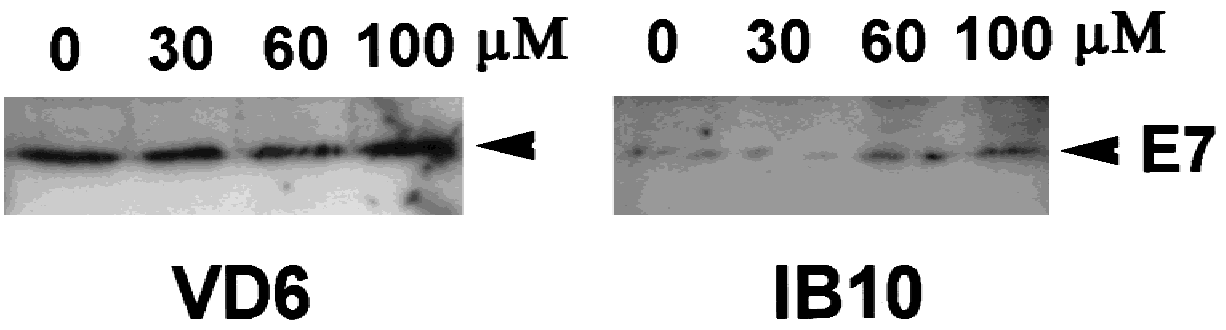

Fig. 2. Western blot analysis of DRB-treated CaSki extracts with VD6 and IB10. The CaSki cells were treated with DRB for 2 hours at the indicated concentrations. E7 expression was down regulated at DRB concentrations greater than 100 μM or when the cells were incubated with DRB for longer than 2 hours.

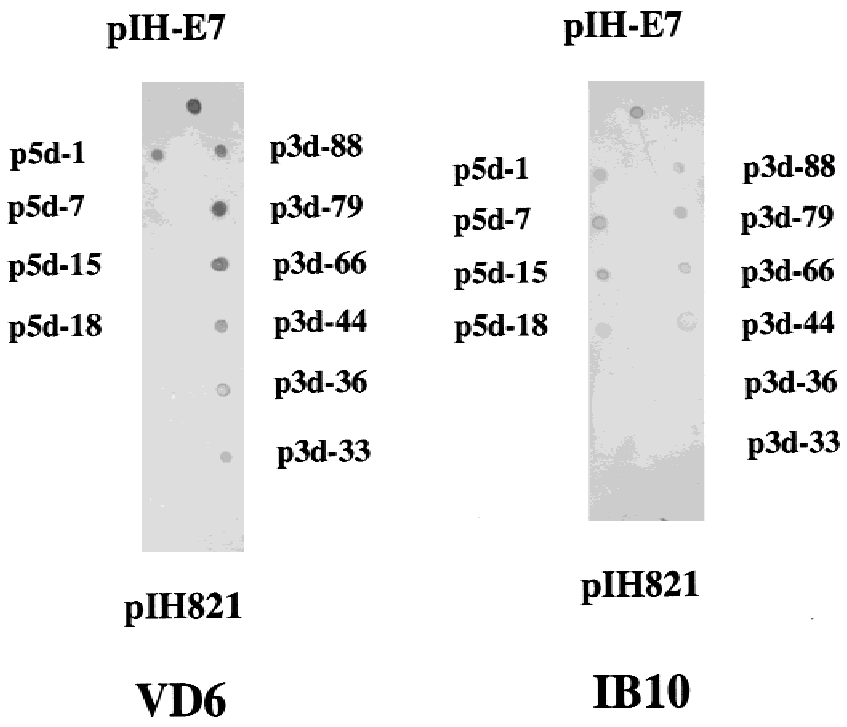

Fig. 3. Dot blot immunoassay with VD6 and IB10 using deletion mutants of E7 protein to estimate its antigenic determinants. Whole cell extracts of *E. coli* expressing full-length E7 or E7 deletion mutants were used as antigens in this assay. Results were confirmed by ELISA or Western blot analysis using the same antigens (data not shown).

single methionine residue. For IB10, the 3′ deletion mutants p3d-36 and p3d-33 did not react (Fig. 3). These results show the sequential nature of the antigenecity of these Mabs. The antigenic site of VD6 must be located in the N-terminal region of the E7 protein and that of IB10 must be located in the middle portion of E7, between amino acids 28 and 44 (based on the assumption that the antigenic site would be a maximum of ten amino acids). Since E7 protein is expressed as a phosphoprotein in CaSki [Smotkin and Wettstein, 1987] and serine residues 31 and 32 are its phosphate acceptor sites [Firzlaff et al., 1991; Barbosa et al., 1990], the antigenic difference between IB10 and VD6 could be due to post-translational phosphorylation of E7 in the eukaryotic system.

In Vitro Phosphorylation and IB10 Reactivities

To investigate the effect of phosphorylation on the reactivity of IB10, the MBP-E7 fusion protein was phosphorylated with casein kinase II (CKII) and [γ-^{32}P]-ATP. Previous studies have shown that E7 is a CKII substrate and suggested that serine residues 31 and 32, in the acidic amino acid stretch of E7, are the phosphorylation sites [Barbosa et al., 1990]. Therefore, we believe that the specific phosphorylation of E7 in the experiment was due to the phosphorylation of these phosphate acceptor sites (Fig. 4A). The in vitro phosphorylation assay over time showed that as the incorporation of phosphate in to E7 increased, the reactivity of the phosphorylated E7 to IB10 decreased. The reac-

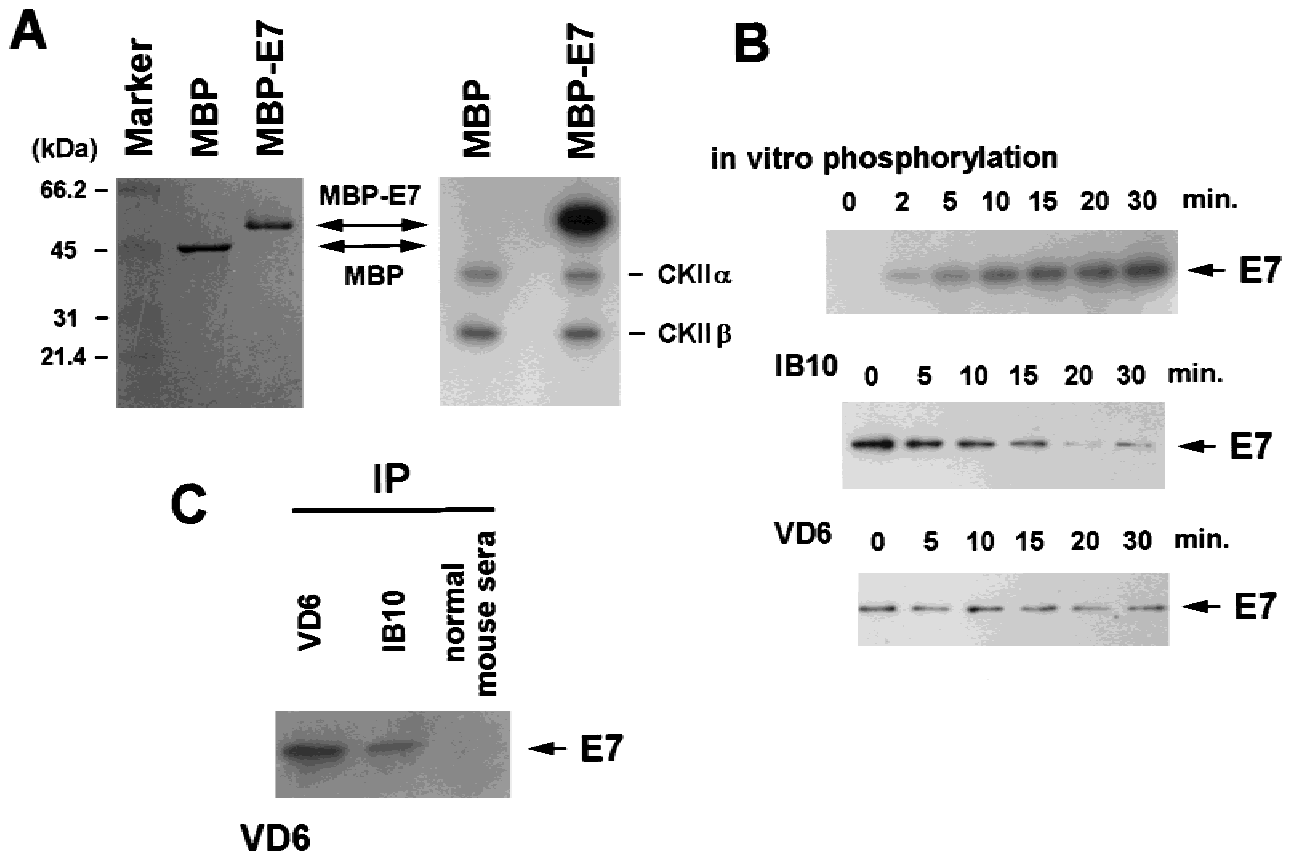

Fig. 4. **A:** Comassie staining of protein (500 ng) used in the phosphorylation assay (left) and of phosphorylated MBP-E7 fusion protein. This CKII kinase reaction was performed for 30 minutes (right). **B:** Phosphorylation of E7 over time and Western blot analysis of phosphorylated E7 protein with VD6 and IB10. **C:** Western blot analysis of immunoprecipitated E7. The E7 protein in CaSki extracts was immunoprecipitated using VD6, IB10, and normal mouse sera. These immunoprecipitates were analyzed by Western blot using VD6. Western blot analysis of these precipitates with IB10 revealed no reactive bands (data not shown).

tivity of VD6 was unchanged over the same time course (Fig. 4B). These results show that the reactivity of IB10 to the middle portion of E7 protein is affected by phosphorylation, particularly under denatured condition such as those in a Western blot assay.

Immunoprecipitation

To assess the reactivities of IB10 and VD6 with E7 protein under native conditions, CaSki cell extracts were immunoprecipitated with VD6 IB10, and normal mouse sera in separate reactions. Western blot analysis of these precipitates using VD6 revealed that both VD6 and IB10 precipitated the E7 protein, although the IB10 precipitated rather weakly (Fig. 4C). The E7 protein precipitated with IB10 was not nonphosphorylated because Western blot analysis using IB10 could not detect E7 in either VD6 or IB10 precipitates (data not shown). It is concluded that the phosphorylated and nonphosphorylated forms of the E7 protein are antigenically cross-reactive under native conditions.

DISCUSSION

Two hybridoma cell lines that secrete antibodies with different reactivities against the E7 protein were established. Although these Mabs were equally reactive to bacterially expressed proteins, their reactivities to E7 expressed in the eukaryotic cell line CaSki were markedly different. These differential reactivities were not due to the conformation of E7, because both antibodies reacted strongly to the bacterially expressed protein by Western blot analysis. HPV16 E7 is phosphorylated on serine residues [Smotkin and Wettstein, 1987] and the resulting negative charge on serine is thought to be important for E7-mediated transformation [Fitzlaff et al., 1991]. Some researchers, however, have reported that this phosphorylation event is not significant [Edmunds and Vousden, 1989; Phleps et al., 1992]. Given that this kind of post-translational modification is restricted to the eukaryotic system, our bacterially expressed protein would have properties different to those of the native E7 protein. We, therefore, investigated whether the phosphorylation of E7 could explain the differences in antigenecity obtained with these antibodies.

Like adenovirus E1A and SV40 T antigen, the E7 ORF is divided into three regions (CRI, CRII, and CRIII) based on their functional characteristics [Moran and Mathews, 1987]. The immunogenic regions of E7 are located in CRI (the N-terminal region) and CRII (the middle portion) [Krchnak et al., 1990; Dillner,

1990; Stacey et al., 1994]. To date, these immunogenic regions (particularly CRII) have yet to be defined precisely. Mabs VD6 and IB10 were reactive with the N-terminal region and with the middle portion of E7, by our crude antigenic estimation. Stacey et al. [1994] have shown that hyperimmune sera against a synthetic peptide (designated 145-4 in the original paper) that spans amino acids 24–38 of E7 could not, or at best weakly, immunoprecipitated Baculovirus-expressed E7 protein. Considering this peptide has a potential phosphorylation site, we believe that this result supports our data. Because the antigenic sites of IB10 are probably located between amino acids 28 and 44 of E7, the epitope of IB10 likely falls within this peptide sequence. Moreover, the peptide antigen and bacterial protein both lack post-translational modification, such as phosphorylation. Therefore, the weak reactivity of these antibodies to expressed E7 eukaryotically results likely from the phosphorylation status of the E7 protein used in the experiment. Phosphorylation of neutral amino acids, such as serine, threonine and tyrosine, is known to affect protein structure and antigenicity [Johnson et al., 1996; Wang, 1988]. Such effects can, in turn, lead to changes in the biologic features of a protein, such as its enzymatic functions. The effect of phosphorylation on E7 antigenecity was therefore investigated using various systems and showed an inverse relationship between E7 phosphorylation and IB10 reactivity (Fig. 4). Mab VD6, which reacted with the N-terminal region of E7, was unaffected by E7 phosphorylation.

Another serine residue (Ser-71) in E7 protein is thought to be a phosphorylation site [Storey et al., 1990]. Although this residue might be phosphorylated, it did not influence the reactivity of IB10, because the antigenic estimation of IB10 indicates that this residue is outside the antigenic sites for IB10. Moreover, the amino acid sequence surrounding ser-71 is not a recognition sequence for a CKII substrate [Kuenzel et al., 1987]. It is concluded, therefore, that phosphorylation of serine 31 and 32 influences the antigenecity of E7. It should be noted that the immunoprecipitation data with these monoclonal antibodies (VD6 and IB10) suggest that there is some cross-reactivity between phosphorylated and nonphosphorylated E7 protein under native conditions (Fig. 4C). It is considered that the structure of E7 influences significantly E7 antigenicity and that this relationship warrants further investigation.

ACKNOWLEDGMENTS

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